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HORMONAL EFFECTS ON PERIOSTEAL MICROVESSELS: A MICROVASCULAR CAST STUDY

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TECHNICAL REVIEW AND APPROVAL

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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INTRODUCTION

Hormone treatment of animals and man can result in either an increase or decrease in bone formation. Parathyroid hormone, administered in low doses, increases bone volume in the rat, dog, and man [1-7]. The enhancement of bone formation at low dosage levels contrasts with bone resorption found either in primary hyperparathyroidism or upon administration of high dosages of parathyroid hormone [8-10]. Prostaglandins E_1 and E_2 cause hyperostosis in young dogs [11] and human infants [12]. Conversely, treatment with glucocorticoids, while transiently increasing bone formation by increasing bone cell maturation [13], ultimately leads to a decrease in both bone cell proliferation and bone matrix synthesis [13,14].

The effect that hormones may have on either the direct or the indirect role of bone microvessels in increasing or decreasing bone formation is poorly understood. However, it is known that prostaglandin E_1 enhances the proliferation of aortic endothelial cells in cell culture [15] and that the ischemia associated with some forms of aseptic necrosis of the femur can be attributed to chronically high glucorticoid secretion [16].

Recent developments in the isolation and culture of bone microvessels [17,18] may make it possible to assess both the importance of capillary endothelial cells to bone cell growth and formation of bone and the sensitivity of endothelial cells to bone hormones and growth factors. As an outgrowth of our interest in bone capillary endothelial cells, we have applied

a microvascular corrosion cast technique [19-22] to study the microvascular patterns of the periosteum of the parietal bone in the rapidly growing rat. We report that both parathyroid hormone and prostaglandin E_1 increased the number and changed the shape of some of the microvessels of the parietal bones and that dexamethasone can either increase slightly or decrease the number of microvessels of the periosteum, depending on the dose and duration of the treatment.

MATERIALS AND METHODS

Sprague-Dawley rats, 2 to 4 weeks old, were used for this study. Usually, litters of 10 rat pups (4 controls, 6 experimentals) were injected once or twice daily for 7 days with parathyroid hormone (PTH, synthetic rat sequence 1-34;) that had a purity of approximately 97% and a peptide content of approximately 68% (Sigma Chemical Co., St. Louis, Mo.). The experimental rats received a dosage of 100 ng PTH in 0.1 ml phosphate buffered saline (PBS), injected subcutaneously over the parietal bone. The control pups were injected with an equal volume of PBS. Prostaglandin E_1, 10 ug in 0.1 ml of 10% ethanol in PBS or vehicle was injected daily for 7 days. Dexamethasone, 0.1 mg daily for 4 or 7 days or 0.01 mg daily for 14 days in 0.1 ml of 10 % ethanol in PBS was injected according to the same litter injection pattern.

Surgical Procedure

The rats were anesthetized by methoxyflurane inhalation and their thoraxes were removed along the mid-axillary lines. Bilateral incisions were begun inferior to the liver and extended sufficiently superior to expose the thymus gland, which was dissected away to better see the aortic root and the innominate and carotid arteries. The heart was elevated with forceps, and an 18 gauge needle was used to penetrate the posterior aspect of the left ventricle adjacent to the posterior coronary artery. Intramedic polyethylene tubing (PE 60) was placed over a 20-gauge needle and used as a cannula, inserted through the left ventricle into the ascending aorta, or in some cases, through the brachiocephalic artery and into the right common carotid artery. The needle was attached to a 213 cm Cutter i.v. line attached to a 60 cc syringe containing 45-65 cc of the perfusate, PBS containing 200 ug/ml

heparin, 0.4 ug/ml isoproterenol, and 0.148 ug/ml papaverine.

The perfusion was done using a Harvard infusion/withdrawal pump. In 4-week-old rats the perfusate was delivered at 11.5 ml/min; in the 3-week-old rats at 9.6 ml/min. At the outset of perfusion, an incision was made in the right auricle to allow an effluent flow of blood. Approximately 10 ml of plastic (Ladd Industries, Inc., Burlington, VT) was prepared (8 g Mercox resin, 2 g monomethyl methacrylate, 0.2 g of benzoyl peroxide catalyst)[19] immediately before use and injected manually through the cannula already in place. The rats were then placed aside for a minimum of 30 minutes to allow the plastic mixture to polymerize.

To attain adequate fixation in order to carryout light microscopy on cross sections of the calvaria, 2 control and 2 experimental rats from each treatment group were perfused with fixative (2% paraformaldehyde, 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2), at 14 ml/min between the initial perfusion and the injection of plastic. When the calvaria were removed from the rats, they were fixed further and simultaneously decalcified using Bouin's fixative. Each calvarium was cut into 3 pieces, dehydrated in an ethanol series, embedded in JB4 resin (Polysciences, Inc., Malvern, PA) and polymerized at room temperature. Microthin sections were prepared using a JB4A microtome, stained with toluidine blue and basic fuschin, and viewed and photographed using a Nikon Labflot Microscope at 32X or 128X.

Digestion of Tissue

To digest away the cells and soft tissues from the microvascular casts, the calvaria were dissected free and placed in 60 mm culture dishes with 8 ml of culture medium containing 3 mg/ml crude dispase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and 0.5 mg/ml collagenase (Worthington Biochemicals, Freehold, NJ) for approximately 16 hours at 37° C. The parietal bones were separated and placed in 3% potassium hydroxide for 48 hours to complete the digestion. The individual parietal bones were transferred to PBS for 8-10 hours, and partially dehydrated in 50% and 70% ethanol for 10 minutes each or briefly rinsed in distilled water and air dried. The specimens were mounted on stubs and coated with gold/palladium in a Hummer V vacuum evaporator. Scanning electron micrographs were taken at 40-1000 X using a Jeol JSM-35 CF scanning electron microscope operating at 20 kv.

RESULTS

Most SEM preparations used the right parietal bone of the rat calvarium because (1) the mid-lateral area of the bone was most consistent for complete filling of the microvascular covering of the outer periosteal surface of the parietal bone and (2) the mid-lateral region of the parietal bone was relatively flat, and microvessels covering this portion of the bone were continuous and were supported during enzymatic digestion and corrosion by the large number of microvessels of the adjacent temporalis muscle. This mid-lateral region was used exclusively to assess the differences in microvessel length and area. Figures 1-6 from PBS-injected or PBS/10% ethanol control specimens show the overall pattern of vascularization of the parietal bone in the young, 3- to 4-week-old rat. As indicated in figures 1 and 2, both small arteries and small veins run within the outer fibrous layer of the periosteum. The rich capillary bed serving the preosteoblasts and osteoblasts of the cellular periosteum is made up of a highly reticular network of capillaries (figures 1-6). Occasionally lying next to the bone surface are either capillaries or more often small venules, which run along and then disappear into the underlying mineralized bone matrix. Such microvessels act as nutritional vessels for the inner portions of the developing bone and the bone marrow.

As shown in figures 7-9, parathyroid hormone treatment (200 ng/day for 7 days) appears to increase the number of capillaries forming the reticular network. In addition, the venules lying along the bone surface increase both in length and in number. At a dosage level of 100 ng/day for 7 days there were no discernable increases in the number of microvessels.

Similar to parathyroid hormone, prostaglandin E_1 (1 ug/day for 7 days), increases the number of capillaries and the number of venules coursing along the bone. In some areas of the bone this affect on the number of venules present is dramatic, seen both by SEM and light microscopy of cross-sections of perfused and fixed specimens (figures 10-12).

Dexamethasone given in high doses, for 4, 7, or 14 days has different effects on both bone formation and microvascularization. At the dosage of 0.1 mg/day for 4 days, there appears to be both a stimulation of bone formation (as found with light microscopy) and microvascularization at 1 week, whereas the same dosage given to rats for 7 days continuously resulted in no change. At the dosage level of 0.01 mg/day for 14 days there appeared to be a slight decrease in the number of capillaries and venules present (figures 13-18).

DISCUSSION

This study demonstrates that at least part of the effect of hormones on bone is on the microvasculature of bone. The recent report that cells possessing the most parathyroid hormone receptors are perivascular cells, perhaps preosteoblasts [23], suggests that more attention needs to be focused on the microvasculature and associated cells. Future schemes to study the control of bone cell development and differentiation, including investigation of growth factors responsible for bone cell proliferation, may well begin with the endothelial cell of the capillary.

Prior to this study using the microvascular corrosion cast technique in bone, most of the previous studies had utilized soft tissue [19-21]. The one published study on bone was more a gross description of small artery and vein distribution to the rat vertebral column [22], than an investigation of the microvascular distribution in the rat vertebra. Inasmuch as our first attempts using this technique failed miserably because of the very rapid polymerization of the Mercox plastic, revision of the procedure, adding monomethyl methacrylate [19] to slow down the rate of polymerization, was vital. By trial and error, we found that the formulation we settled upon allows approximately 5 minutes from the time the plastic components are mixed until they must be injected. The smaller the needle and catheter used, the more critical the time factor.

The microvascular cast corrosion technique appears to be more difficult in bone or other dense connective tissues as compared to soft tissues such as lung [19] or gut [20]. Large muscle masses or large tendons adjacent to or

attaching to bone makes unimpeded study of the general microvasculature of the bone surface difficult. Furthermore, use of the 10% KOH recommended for soft tissue corrosion can be counterproductive. Once the tendinous or muscle attachments is eaten away, the entire microvascular cast of the muscle breaks free from the bone, taking with it the periosteal microvessels, many of which originated from and attached to their adjacent muscle microvessels. This problem can be eliminated in part by trimming the muscle attached to the bone to decrease the muscle mass and by pre-treating the bones with dispase and collagenase and then with corrosive potassium hydroxide, at 3% rather than 10%.

Perfect perfusion and infiltration with plastic of all microvessels in the entire parietal bone was difficult and rare. When the tip of the catheter was placed in the ascending root of the aorta close to the roots of the brachycephalic and left common carotid arteries, the entire head was perfused. Sacrificing the area of plastic infiltration for consistency, we inserted the catheter into the right common carotid, just superior to the bifurcation of the brachycephalic artery. This alteration improved consistency to the degree that the microvasculature of the midlateral area of the parietal bone was routinely well filled, allowing an attempt to quantitate the increase or decrease in the microvascularity of the parietal bone following hormonal treatment. Similarly, catheterization of the descending thoracic aorta allowed our investigation of the microvasculature of the tibia.

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LEGENDS

Figure 1. Control, low-power, survey scanning electron micrograph showing the general distribution of microvessels over the parietal bone of the young rat. X40.

Figure 2. Control, scanning electron micrograph of the microvessels of the parietal bone exhibiting the presence of small arteries (A), small venules (V), and capillary loops (C). X100.

Figure 3. Control, light microscopy of a cross-section through the parietal bone. Microvessels of various sizes can be seen within the periosteum (P). X64.

Figure 4. PTH-treated, scanning electron micrograph showing a general increase in number of microvessels. X40.

Figure 5. PTH-treated, scanning electron micrograph exhibiting an increase in the number of small venules. X100.

Figure 6. PTH-treated, scanning micrograph showing the imprint of endothelial cell nuclei along the casts of the capillary network. X500.

Figure 7. PTH-treated, light microscopy of a cross-section of the parietal bone showing the general increase in microvessels in the periosteum (P). X64.

Figure 8. PGE_1-treated, scanning electron micrograph showing a large increase in the number of microvessels covering the parietal bone. X40.

Figure 9. PGE_1-treated, scanning electron micrograph exhibiting a great increase in the number of microvessels, particularly capillary loops. X100.

Figure 10. PGE_1-treated, scanning electron micrograph showing an increase in microvessels of all sizes. X100.

FIGURE I

FIGURE II

FIG. 3

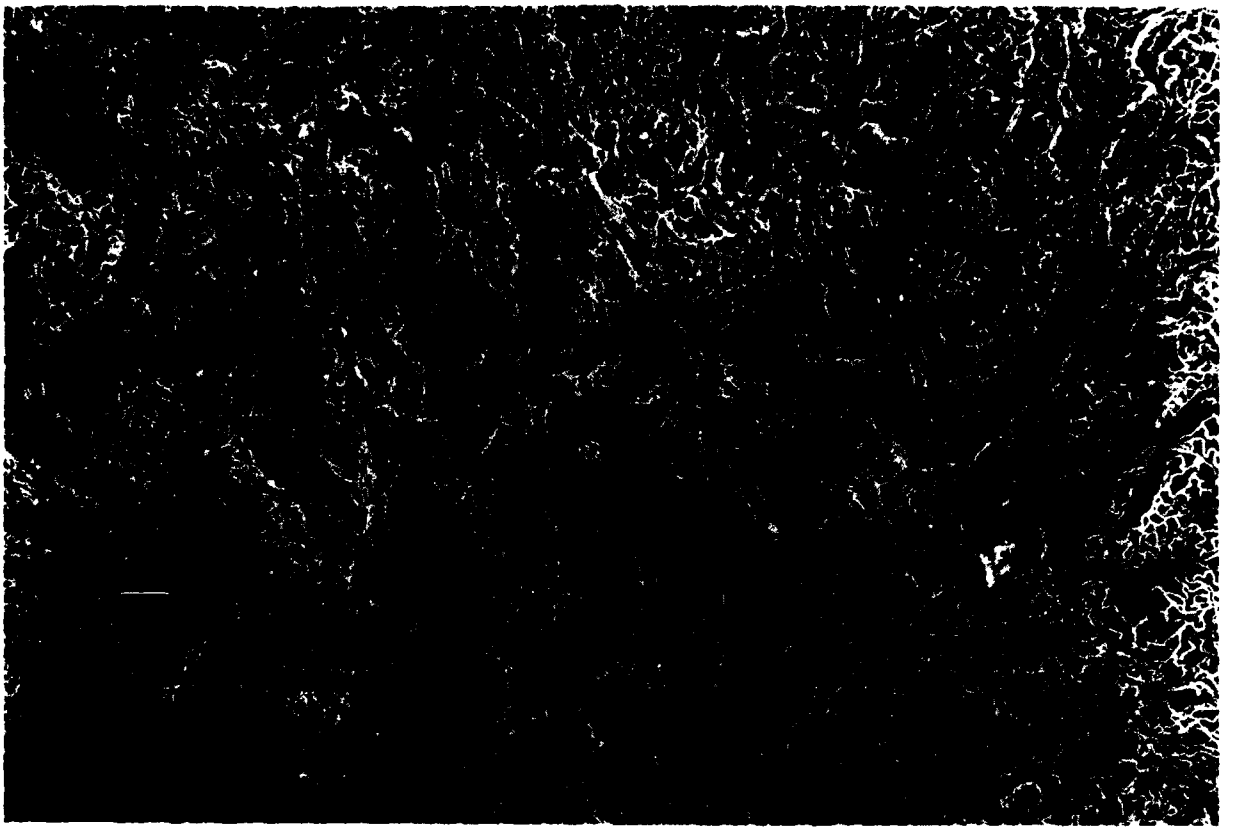

FIG. 4

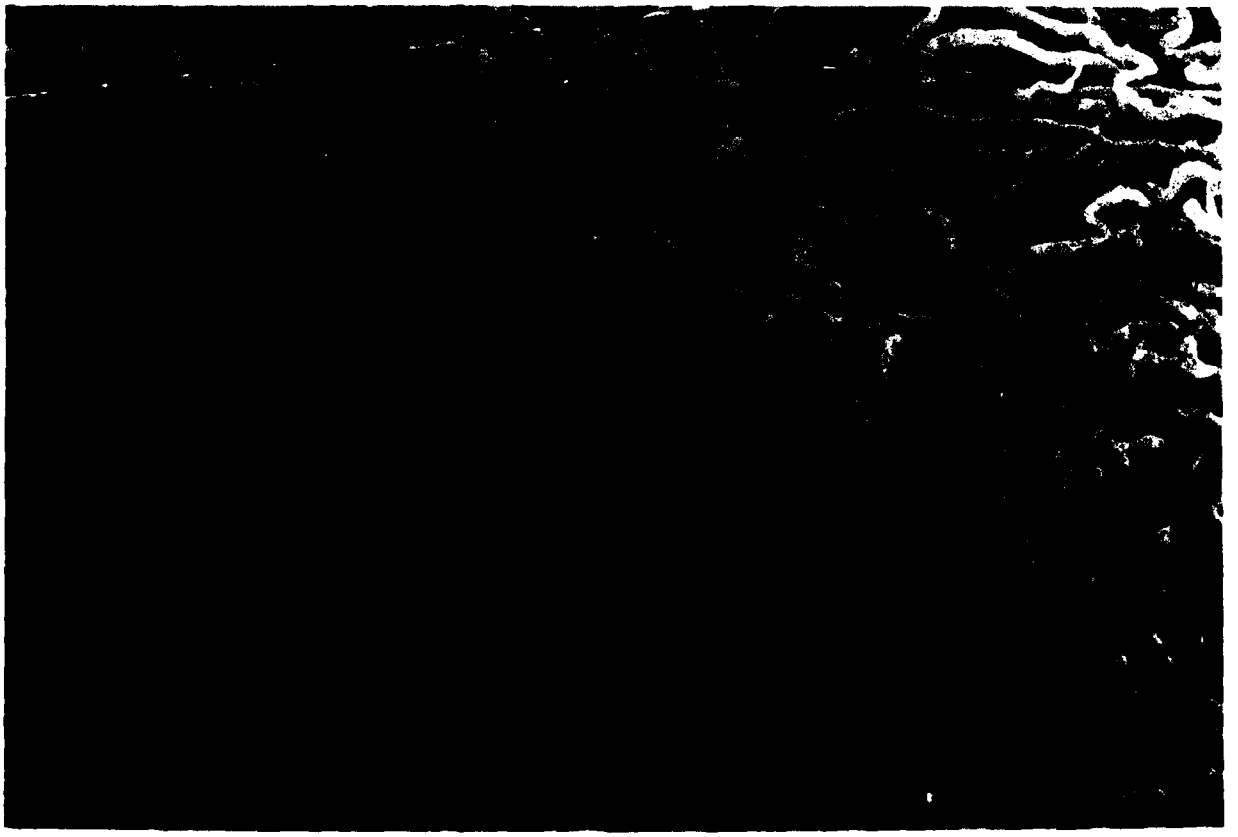

FIG. 6

FIG. 7

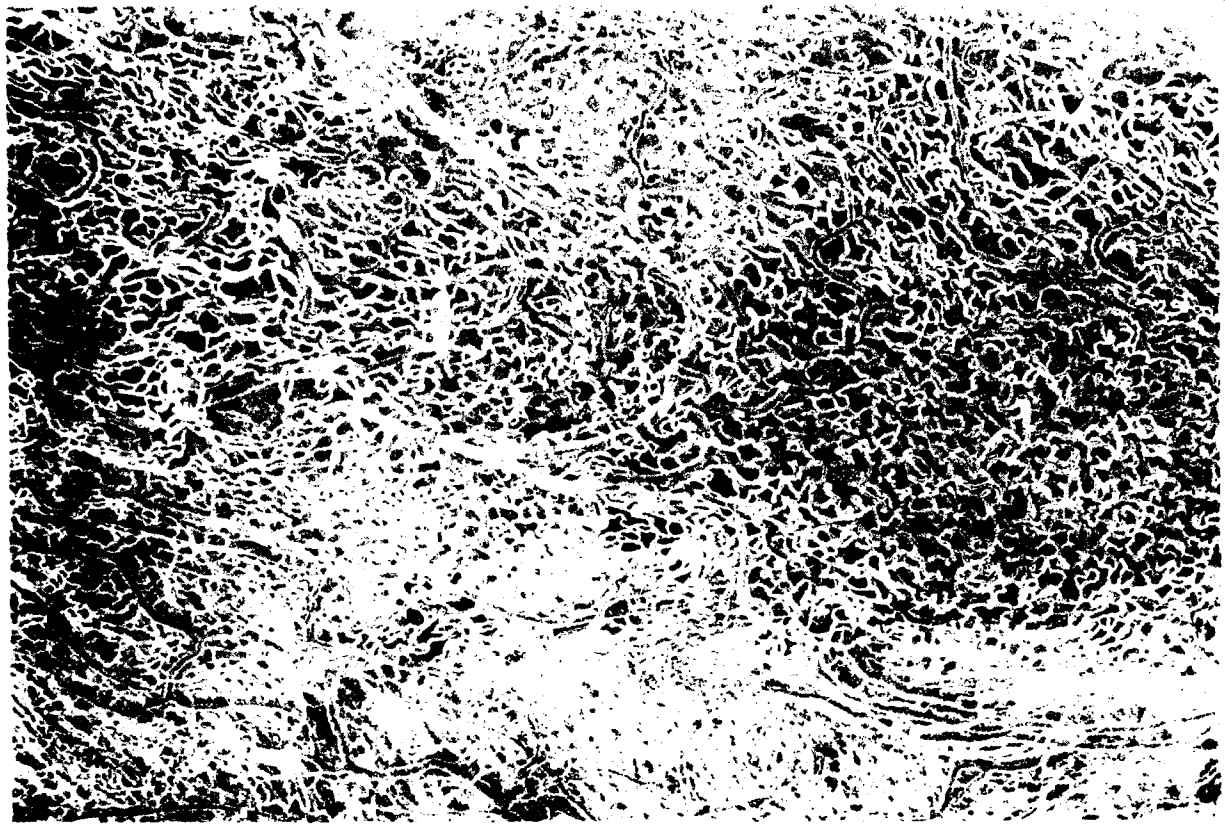

FIG. 8

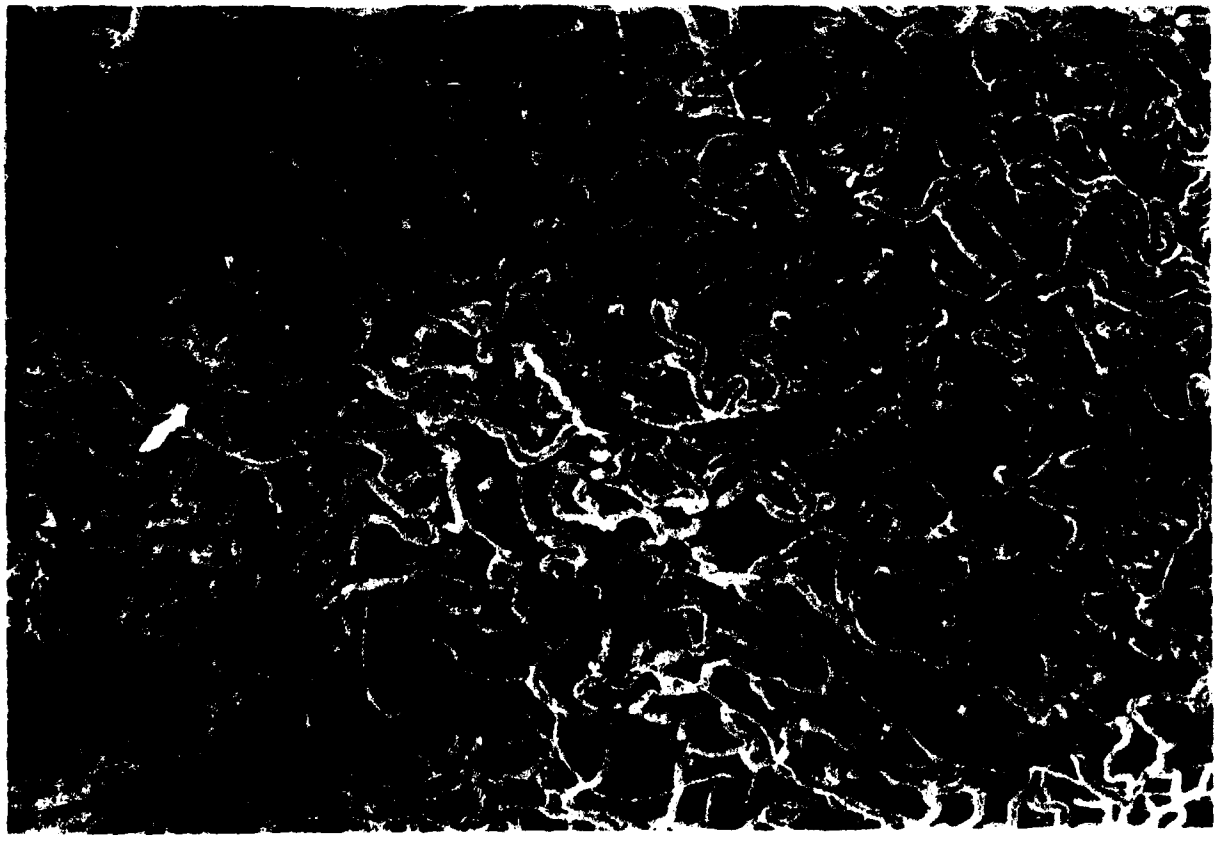

FIG. 9

FIG. 10